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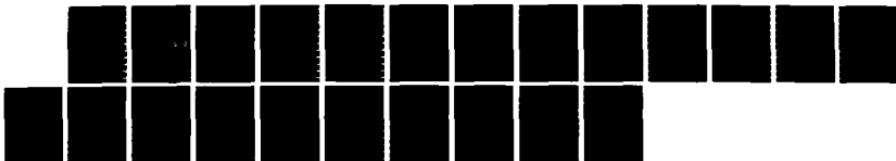
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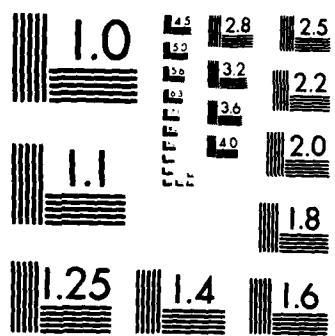
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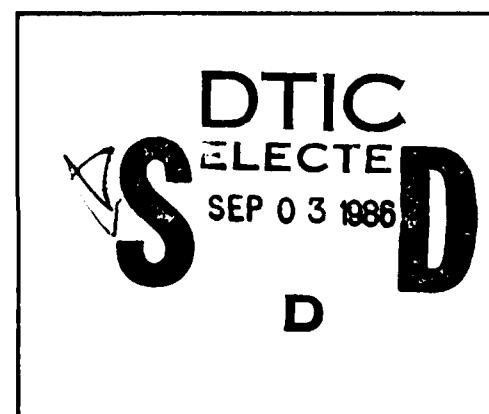
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**Experimental Design and Methods for Development of Diagnostic  
Assays for Schistosomiasis Using Monoclonal Antibodies**

**Final Comprehensive Report**

**Mette Strand, Ph.D.**

**August 25, 1983**

**Supported by**

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)  Monoclonal antibodies have been prepared as probes for rapid, simple, accurate diagnostic assays for schistosomiasis.  These monoclonal antibodies are being characterized with respect to: (1) biological relevance (elicitation of an immune response in the infected host against same antigenic target); (2) relevance to stage of infection		

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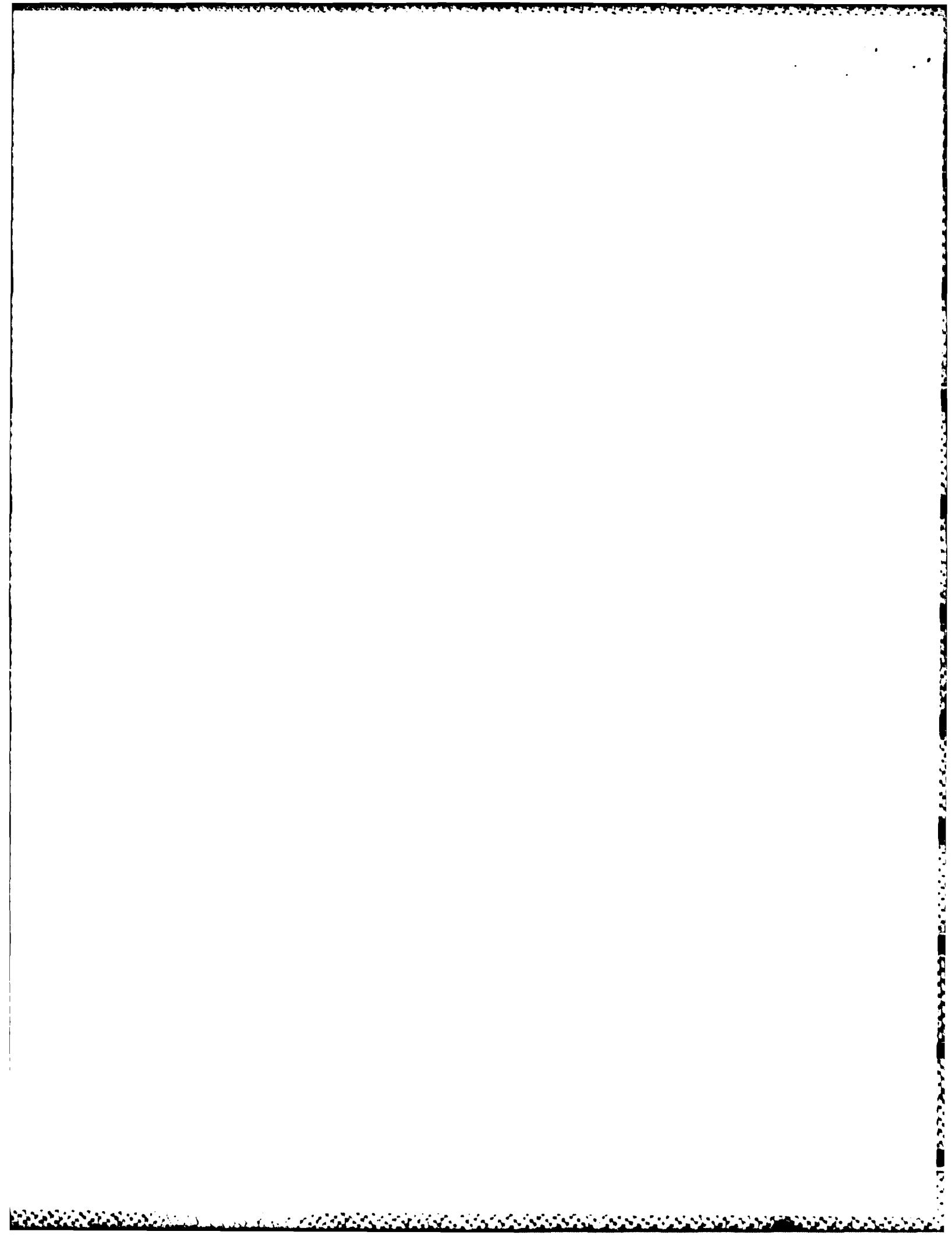
(monoclonal antibodies specific for acute and chronic infections); (3) lack of cross-reactivity with other parasitic antigens; (4) specificity for S. mansoni, S. haematobium and S. japonicum; (5) detection of antigen(s) in the circulatory system.

The objective of this study is to establish two diagnostic assay systems: (1) Early detection of schistosomal infection by assay of patients antigen level or antibody response; (2) a quantitative assay of antigenemia as an index for the therapeutic efficacy of drug treatment.

These studies will provide monoclonal antibody probes for diagnosis of schistosomiasis and for prevention of infection by detection of infectious organisms contaminating water sources.

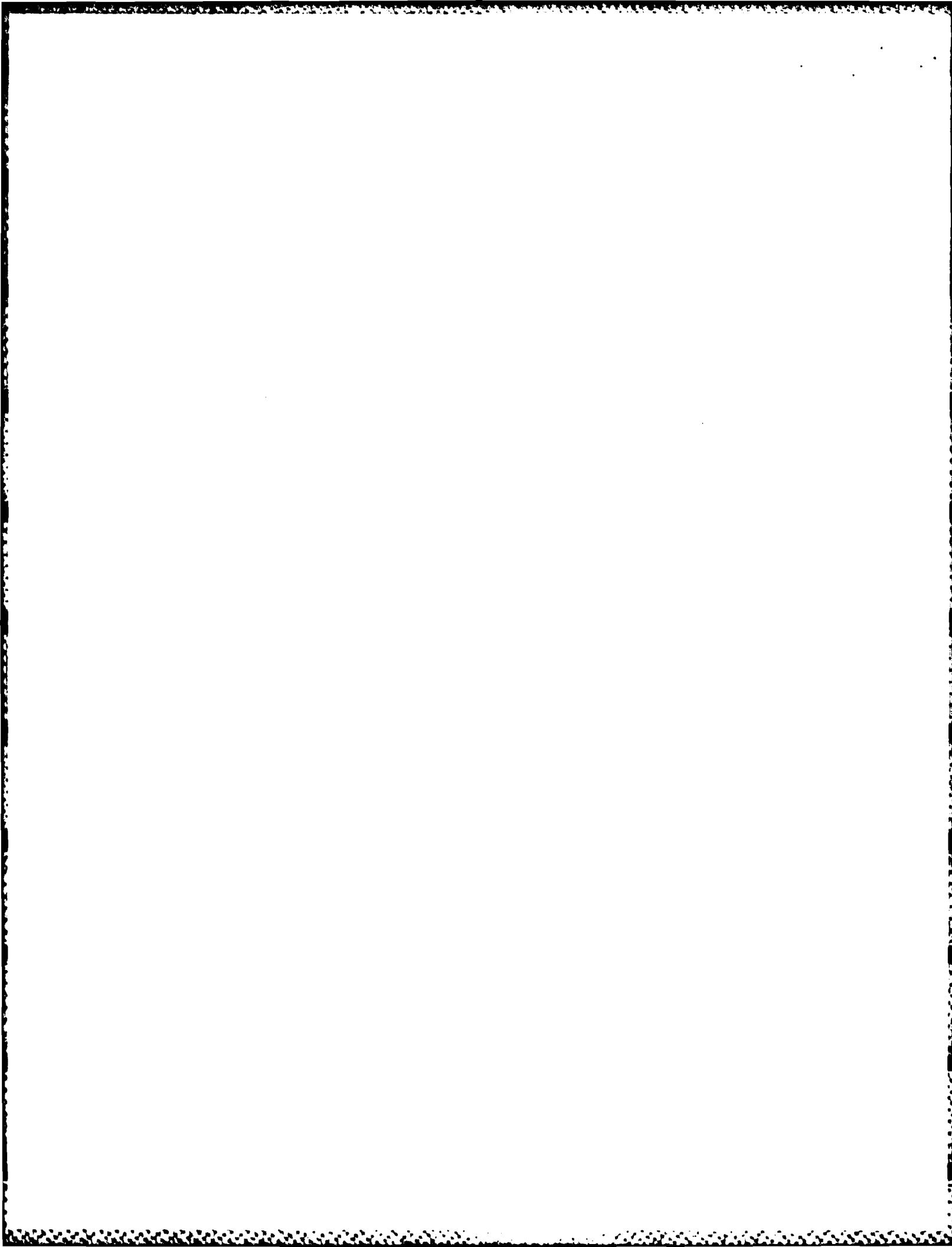
#### FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).



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## I. OBJECTIVE OF RESEARCH PROPOSAL.

The objective of this proposal was to develop rapid, accurate, and simple diagnostic assays for schistosomiasis by use of monoclonal antibodies.

## II. BACKGROUND.

Parasitic infections are present throughout the world, not only causing a major public health problem, but also an economic loss to the endemic countries. Of the many debilitating parasitic diseases, schistosomiasis ranks as one of the most widespread tropical infections affecting an estimated 300,000,000 individuals.

The schistosome is an exquisitely evolved parasite that presents dozens of antigens to the infected host. These antigens include components which derive from infecting cercariae, from developing schistosomula, from adult worms, and from maturing eggs. Among these antigens are presumably those which elicit the immunoprotective response, but they also include the antigens responsible for eliciting the granulomatous inflammation which is the hallmark of schistosomiasis.

None of the wide variety of parasitological and serological tests available to detect infection with Schistosoma and other parasites are sufficiently accurate. The pathologic examination of feces or urine by enumerating the eggs is not a sensitive assay. Classically, serologic techniques have been used to establish whether infection has occurred by measuring antibody levels. However, the immunological assays presently employed are extremely variable due to the crude mixture of proteins that serve as antigens. In addition, difficulties are also encountered with respect to specificity, since schistosome antigens cross-react with antigens of at least five other parasitic pathogens. Sera from patients or experimental animals infected with Schistosoma, Fasciola hepatica, Trichinella spiralis, Taenia solium, Echinococcus granulosus, or Paragonimus westermani cross-react in diagnostic assays with antigens derived from schistosomes, whether as whole organisms (1-4), crude extracts (5-9), partially purified extracts (10-11), or non-schistosome parasite extracts (12-17). Furthermore, mice challenged with S. mansoni after infection with T. spiralis (18) or F. hepatica (19), or after immunization with isolated F. hepatica antigens (20-23) or P. westermani antigens (17) have a significantly reduced S. mansoni worm burden. Conversely, mice infected with S. mansoni for over 28 days significantly resisted an F. hepatica challenge (19,24). Although these previous studies have established the existence of parasitic cross-reactivity, little is known about the molecular identity of these Schistosoma antigens.

Furthermore the three major species of human schistosomes (S. mansoni, S. haematobium, and S. japonicum) exhibit extensive serological cross-reactivity despite numerous morphological and physiological dissimilarities. Sera of patients infected with heterologous Schistosoma species are cross-reactive, irrespective of the schistosome developmental stage used in diagnostic assays; be it worms (25-29), eggs (6,28,30-36) or cercariae (3,4).

The serologic assays are also subject to variation depending upon the source of antigen (cercaria, worm, or egg), method of solubilization, and method of coating antigen to polymeric surfaces (micro-ELISA and StiQ<sup>TM</sup> assay). Such untreated surfaces selectively bind neutral proteins, thus, cathodic or anodic antigens bind weakly, if at all. We have observed that several of the antigens which we have identified and which are immunoreactive with serum of infected humans require pretreatment of the microtiter plates prior to binding (37,38). In addition, use of crude antigen extracts can result in false negative antibody levels, since the stage of infection (acute versus chronic) requires different antigens for reactivity (see below).

### III. APPROACH TO RESEARCH PROPOSAL:

Our major goals are:

- (1) to identify the proteins of Schistosoma that elicit an immune response in infected humans;
- (2) to prepare monoclonal antibodies against these proteins;
- (3) to relate the presence of specific schistosomal proteins to the stage of infection;
- (4) to determine the cross-reactivity of schistosomal antigens with antigens of other parasites; and
- (5) to develop assay systems for the measurement of schistosomal antigens and antibodies.

Immunoassays based on monoclonal antibodies as defined chemical reagents can be expected to be reproducible and standardized. In addition, such reagents permit identification and purification by immunoaffinity chromatography of antigens useful as probes for diagnosis of distinct stages of infection. For example, by use of schistosomal antigens that are uniquely expressed only in cercariae or schistosomula it might be possible to diagnose acute infections. Conversely, a quantitative assay of antigenemia utilizing defined monoclonal antibodies may provide an index for the therapeutic efficacy of drug treatment of chronically infected patients.

### IV. RESULTS

During the 8 months in which we have been funded for this research our efforts have been concentrated in four areas: (a) Tegumental expression of major surface glycoproteins; (b) Identification of species-specific and gender-specific proteins and glycoproteins of three human schistosomes; (c) Gender-specific and pair-dependent glycoproteins antigens of Schistosoma mansoni; and (d) Schistosoma: Identification of genus-, species-, and gender-specific antigenic worm glycoproteins.

#### A. Tegumental Expression of a Major Schistosome Structural Glycoprotein.

Changes in the schistosome tegument are postulated to be one of the mechanisms by which schistosomula become refractory to immunologic defense mechanisms of the mammalian host. Observed morphologic changes in the tegument include the loss and reappearance of surface spines, the elaboration of a heptalaminate surface membrane, and a change in the pattern of intramembrane particles. The adsorption of host antigens onto the developing tegument has also been reported.

We are studying the antigenic composition of the schistosome tegument using monoclonal antibodies to elucidate the mechanisms whereby schistosomes evade the host's immune defenses.

##### 1. Tegumental Expression in Larval and Adult Stages of Major Structural Glycoprotein

We have used a monoclonal antibody to identify and characterize an antigen of approximately 180,000 daltons that elicits an immune response in infected hosts. This antigen appears to be a major structural surface glycoprotein of cercariae, schistosomula, and adult worms of both sexes. As measured by direct binding assay, the concentration of this glycoprotein in the whole protein extracts was at least ten-fold higher than that of a previously described schistosome glycoprotein antigen (38). Immunofluorescent microscopy revealed an extensive and organized distribution of the antigen on the larval and adult tegument. Binding of directly labeled antibody 132C4A/4 to egg protein extract suggests that this glycoprotein is an important structural component of miracidia as well. In contrast to the spine glycoprotein which is absent from the egg (38), this glycoprotein is expressed continuously by all schistosome developmental stages.

We studied the pattern of this antigen on the surface of larval and adult parasites to determine whether a change in its expression could be correlated with the development of resistance to the immune defenses of the host. Because young larvae are susceptible to immune defenses, we first identified the pattern of the structural organization of this glycoprotein on the surface of fixed and unfixed mechanically derived schistosomula. By indirect immunofluorescence, they exhibited a striking pattern of narrow bands reminiscent of the tegumental folds seen in the scanning electron microscope.

Adult worms of both sexes exhibited a pattern identical to that exhibited by three-hour schistosomula. In addition, the tubercles of the male's dorsal surface were outlined, while the tubercular spines remained unstained. The spine glycoprotein identified by another monoclonal antibody (38) is expressed on the surfaces of cercariae, schistosomula, and adult worms. At least with respect to these two glycoproteins, therefore, the schistosome tegument maintains the same surface antigens throughout development in the vertebrate host.

Development of the heptalaminate double outer membrane is one morphologically evident change considered likely to play an important protective role. The two bilayers composing this structure present different appearances to freeze-fracture scanning electron microscopy. Both adult worms and schistosomula examined after three hours incubation in serum or growth medium exhibit a heptalaminate double outer membrane. Monoclonal antibody 132C4A/4 was derived from a fusion prepared against cercarial glycoproteins; we determined that the glycoprotein it recognized was present on the trilaminate cercarial surface membrane by examining the surface of freshly transformed schistosomula following separation of tail and body. No immunofluorescence was observed immediately following vortex treatment, probably because the cercarial glycocalyx prevented access to the rhodamine conjugated antibody. In contrast, the characteristic pattern of this glycoprotein antigen was apparent as soon as thirty minutes thereafter, and did not subsequently change. Therefore, the trilaminate and heptalaminate surface membranes did not differ with respect to expression of this major antigen. The adult worm's spines also continue to express an epitope identical to that on the cercarial and schistosomular spines (38). These results demonstrate that at least two of the major surface glycoproteins of the outer faces of the trilaminate and heptalaminate teguments are antigenically conserved.

## 2. Lung-Stage Expression of a Major Structural Surface Glycoprotein.

Attempts to demonstrate the presence of the 180,000 molecular weight glycoprotein recognized by monoclonal antibody 132C4A/4 (described above and in detail in 39) on the surface of lung-stage schistosomula were unsuccessful.

We now report (40) that the immunofluorescent pattern obtained with this antibody (RITC-132C4A/4) was enhanced when formalin-fixed worms were dehydrated in ethanol, and that ethanol-treated formalin-fixed lung-stage schistosomula also exhibited positive immunofluorescence with an identical surface distribution for this antigen. The epitope recognized by RITC-132C4A/4 was present on the tegument of *S. mansoni* and *S. haematobium*, but not on *S. japonicum*. Our findings that lung-stage schistosomula are completely blocked by lipids or glycolipids might explain their insusceptibility to immune attack. Young larvae and adult worms, whose antigens are at best only partially blocked, may employ other defenses to insure their survival.

## B. Identification of Species-Specific and Gender-Specific Proteins and Glycoproteins of Three Human Schistosomes.

One of the specific goals of the proposed project was to identify the proteins that elicit an immune response in infected humans. We have previously characterized the cercarial glycoproteins that are reactive with sera of *Schistosoma mansoni* infected patients (37). We have now completed the analyses of adult worm proteins and glycoproteins of *Schistosoma* (41).

The three major species of human schistosomes, Schistosoma haematobium, Schistosoma japonicum, and Schistosoma mansoni exhibit numerous morphological and physiological dissimilarities. These differences include the shape and size of the egg, tuberculation of the male's dorsal tegument, disposition of the reproductive organs, fecundity, intermediate host specificity, and preferred intravascular habitat in the human host. Despite these striking differences, extracts of these parasites show antigenic cross-reactivity in serological assays. Comparison of the protein composition of these three related species may lead to an identification of differences in their structural components and in their antigenicity.

We have developed procedures for analyzing species-specific and gender-specific polypeptides of three species of human schistosomes. Proteins were metabolically labeled with  $^{35}\text{S}$ -methionine to exclude possible contaminating host proteins. Proteins synthesized by separated male and female worms were examined by two-dimensional gel electrophoresis. Since proteins released by schistosomes in membrane fragments or as soluble secretions and excretions may represent important antigens during natural infections we extended the analysis to include proteins solubilized from the labeling media. The identification of species-specific and gender-specific products was greatly enhanced by the isolation of glycoproteins by lectin affinity chromatography. Many of the released (shed or secreted) proteins were glycosylated, and most of the synthesized glycoproteins were in turn released. The most striking species-specific and gender-specific differences were observed in the glycoproteins, of which one was restricted to S. mansoni females, four to S. japonicum females, four to S. haematobium females, and six to S. haematobium males. These studies provide a possible starting point for exploring the molecular evolutionary divergence of these closely related parasites, the occurrence of dioecy in the schistosomatidae, and for the development of improved serodiagnostic reagents.

C. "Gender-Specific and Pair-Dependent Glycoprotein Antigens of Schistosoma mansoni."

As we have demonstrated in the study described above, analyses of the total protein composition of male and female S. mansoni by high resolution SDS-polyacrylamide gel electrophoresis have shown a few sex-specific differences. Whether these differences in protein composition are reflected in the immune response elicited by male and female schistosomes has not been established. We wished, therefore, not only to determine whether the antigens presented by the parasite to the host differed during unisexual versus bisexual infections, but also to assess the host's humoral responses under these different conditions. Since only patently infected mice, in contrast to unisexually infected mice, acquire resistance to re-infection (reviewed by Dean, 42), we reasoned that antigens immunogenic only during patent, bisexual infections (pair-dependent antigens) might be relevant to the immune mechanisms involved in the development of resistance to re-infection. Similarly, because patently but not unisexually infected mice are sensitized for an augmented granulomatous response to injected schistosome eggs, we reasoned that the pair-dependent antigens might play a role in the immunopathogenesis of disease.

Concanavalin-A binding glycoproteins of unisexually and bisexualy reared male and female worms were labeled in vitro with  $^{35}\text{S}$ -methionine or  $^{125}\text{I}$ odine and the two-dimensional maps of their polypeptides compared. The total protein compositions of male and female worms were very similar. In contrast, the patterns of glycoproteins immunoprecipitated by antisera from mice chronically infected with only male, only female, or both male and female worms were distinctive, indicating that the immune response elicited by the glycoproteins of male and female *S. mansoni* varied with the type of infection. Radioiodinated cercarial and egg proteins were also studied. Several polypeptides of cercariae and eggs and eleven of 32 antigenic worm glycoproteins were recognized only by the antiserum from bisexualy infected mice, not by the antisera from unisexually infected mice. These pair-dependent antigens may be relevant to the immune mechanisms involved in pathogenesis and resistance to re-infection. Manuscript entitled "Gender-Specific and Pair-Dependent Glycoprotein Antigens of *Schistosoma mansoni*" by Aronstein and Strand has been submitted (43).

D. *Schistosoma*: Identification of Genus-, Species-, and Gender-Specific Antigenic Worm Glycoproteins.

This study was designed to determine which *Schistosoma* glycoproteins were immunoreactive with sera of patients infected with *Schistosoma* or other parasites in order to identify the genus-specific, species-specific, or cross-reactive glycoproteins. We also wished to determine the immunoreactivity of the recently identified species- and gender-specific *Schistosoma* adult worm glycoproteins (41). For this purpose, glycoproteins of adult male and female worms of the three species (encompassing five strains) of *Schistosoma* were utilized, since it has been shown previously that the glycoproteins are the most immunoreactive components of the protein extracts (37,44-50). This is the first study in which glycoprotein extracts of worms metabolically labeled with  $^{35}\text{S}$ -methionine were used as antigens for immuno- precipitation with sera of patients infected with one of three species of *Schistosoma* or *Trichinella spiralis*, *Taenia solium*, *Echinococcus granulosus*, *Entamoeba histolytica*, or *Wuchereria bancrofti*. The *S. mansoni* glycoproteins that were immunoprecipitated by sera of patients infected with *S. mansoni* were indistinguishable from those precipitated by sera of patients infected with *S. haematobium*. Analogous results were obtained by use of *S. haematobium* glycoproteins as the radiolabeled antigen. In contrast, sera of patients infected with *S. japonicum* or parasites not of the genus *Schistosoma* immunoprecipitated fewer than half of the major *S. mansoni* or *S. haematobium* glycoproteins. Likewise, several *S. japonicum* glycoproteins were cross-reactive, while others exhibited genus-, species- and gender-specificity. The genus- and species-specific immunoreactive *Schistosome* antigens will be discussed below with respect to their relevance for serodiagnosis. Manuscript entitled "Schistosoma: Identification of Genus-, Species-, and Gender-specific Antigenic Worm Glycoproteins" by Norden and Strand has been submitted (51).

## V. DISCUSSION OF RESULTS

Several important results have been obtained from these studies.

### A. Tegumental Expression of Major Surface Glycoproteins.

Several models have been proposed to account for protective changes in the schistosome tegument. These include adsorption of host antigens (52,53), shedding of surface antigens from the developing schistosomula (54-56), or that the schistosome's syncytial tegument itself undergoes an intrinsic change unrelated to changes in its surface antigens that makes it refractory to immune attack (57,58). It was therefore of relevance to study antigenic expression on the surface of schistosome throughout development in the vertebrate host. We have studied several major surface antigens utilizing tetramethylrhodamine (RITC) conjugated monoclonal antibodies. These results demonstrated that at least two of the major surface glycoproteins of the outer faces of the trilaminar and heptalaminate teguments are antigenically conserved (38,39). In addition, we have demonstrated that lung-stage schistosomula are completely blocked by lipids or glycolipids which might explain their insusceptibility to immune attack (40).

### B. Species-specific and Gender-specific Proteins and Glycoproteins of Schistosoma.

As analyzed by two-dimensional gel electrophoresis of metabolically labeled proteins and glycoproteins, the three major human schistosomes exhibited species-specific and gender-specific differences (41). These studies included analyses of not only proteins synthesized by separated male and female worms, but also proteins released by schistosomes in membrane fragments or as soluble secretions and excretions. Such components may represent important antigens during natural infections (9,59-62). The identification of species-specific and gender-specific products was greatly enhanced by the isolation of glycoproteins. A remarkable finding was that many of the *S. haematobium* glycoproteins and most of the *S. japonicum* and *S. mansoni* glycoproteins were released (shed or secreted) into the culture medium. Polypeptides released by schistosomes in culture may be similar to those released into the circulation of infected hosts, where these may play a role in modulating the host's immune response and in the immunopathology of schistosomiasis (63,64).

The genus-, and species-specific glycoproteins as well as those released (circulatory antigens) that we have identified therefore may be valuable for development of specific diagnostic assays for schistosomiasis.

C. Gender-specific and Pair-dependent Glycoprotein Antigens.

The schistosomes are the only dioecious trematodes and exhibit notable sexual dimorphism. Furthermore, the pathological lesions and clinical manifestations of schistosomiasis result from the sexual reproduction of schistosome couples. The mechanisms by which male and female schistosomes mate are unclear (65). Having established the presence of gender-specific schistosome glycoproteins (41), we wished to assess the host's humoral immune response in unisexual and in patent, bisexual infections. We found that patently infected mice exhibit a markedly augmented and qualitatively different response; eleven of 32 metabolically labeled *S. mansoni* glycoproteins reacted only with sera of patently infected mice. These antigens may be involved in the immunopathogenesis of disease; they may also be involved in the protective immune response, since only bisexually infected, and not unsexually infected, mice acquire significant resistance (42).

D. Schistosoma: Identification of Genus-, Species- and Gender-specific Antigenic Worm Glycoproteins.

To date no studies have identified which of the many proteins of *Schistosoma* elicit an immune response in infected patients nor are there data available on the molecular identity of their genus- and species-specificity. We therefore thought it would be relevant to carry out such studies. We have identified 30 major polypeptides of *S. mansoni* that are reactive with sera of patients infected with either *S. mansoni* or with *S. haematobium*. In contrast only half of these polypeptides are recognized by sera of patients infected with *S. japonicum*. In addition, we have identified the polypeptides that are recognized by sera of patients infected with other parasites not of the genus *Schistosoma*. More importantly, we have also identified the polypeptides against which serum of each patient infected with homologous or heterologous schistosome react. Likewise, we have identified the polypeptides which show marked variation in the humoral immune responses of individual patients. These studies have been carried out with *S. mansoni* (Puerto Rican strain) *S. haematobium* (Egyptian and Ghamaian strains) and *S. japonicum* (Chinese and Japanese strains). The identification of these polypeptides will be highly valuable for the development of specific serodiagnostic assays for schistosomiasis.

VI. CONCLUSIONS

The goal of this research is the development of sensitive and specific serodiagnostic assays for schistosomiasis. Immunodiagnostic assays using monoclonal antibodies as reproducible and standardized probes for the quantitation of specific antibodies, schistosome antigens, and antigen-antibody immune complexes can be developed when appropriate antigenic targets are identified. The use of such monoclonal antibody immunodiagnostic assays will permit more effective diagnosis, more accurate epidemiological surveys, and better evaluation of therapeutic interventions.

During the first eight months of funding, we sought to determine if such immunodiagnostic assays are in fact feasible by analyzing the glycoprotein antigens of *S. haematobium*, *S. japonicum*, and *S. mansoni*. As outlined above, we have established that the glycoproteins expressed by these three important human schistosomes include polypeptides that are genus-specific, species-specific, stage-specific, and gender-specific. We have also shown that monoclonal antibodies can be prepared against these antigens. The feasibility of developing the desired serodiagnostic assays has therefore been demonstrated.

VII. RECOMMENDATION

Continuation of funding will permit us to carry through these studies by using monoclonal antibodies recognizing these specific antigens in actual serodiagnostic assays. Carefully controlled murine infections will be used to establish quantitative assays measuring the presence of antigens and antibodies in the circulation. Later, it will be possible to extend these tests to include natural human infections.

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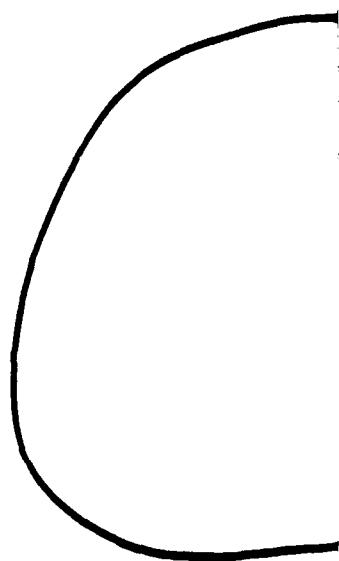
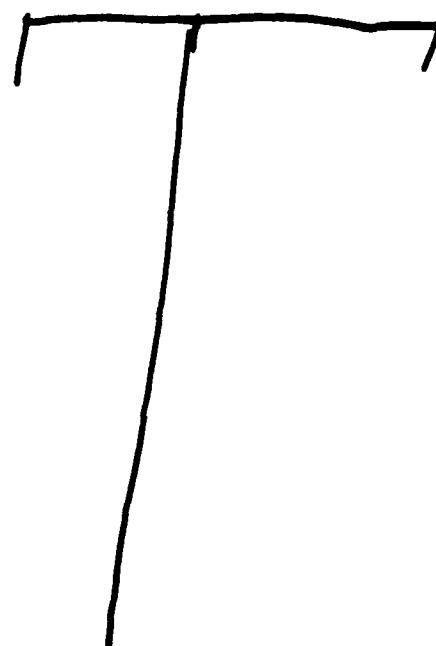
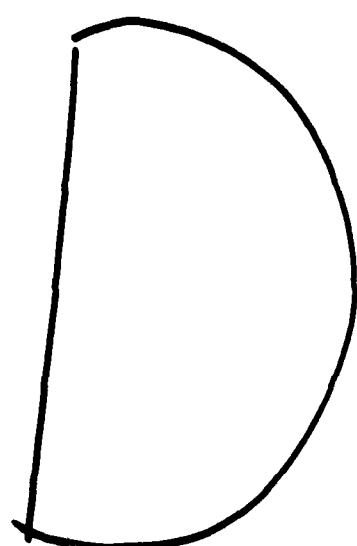
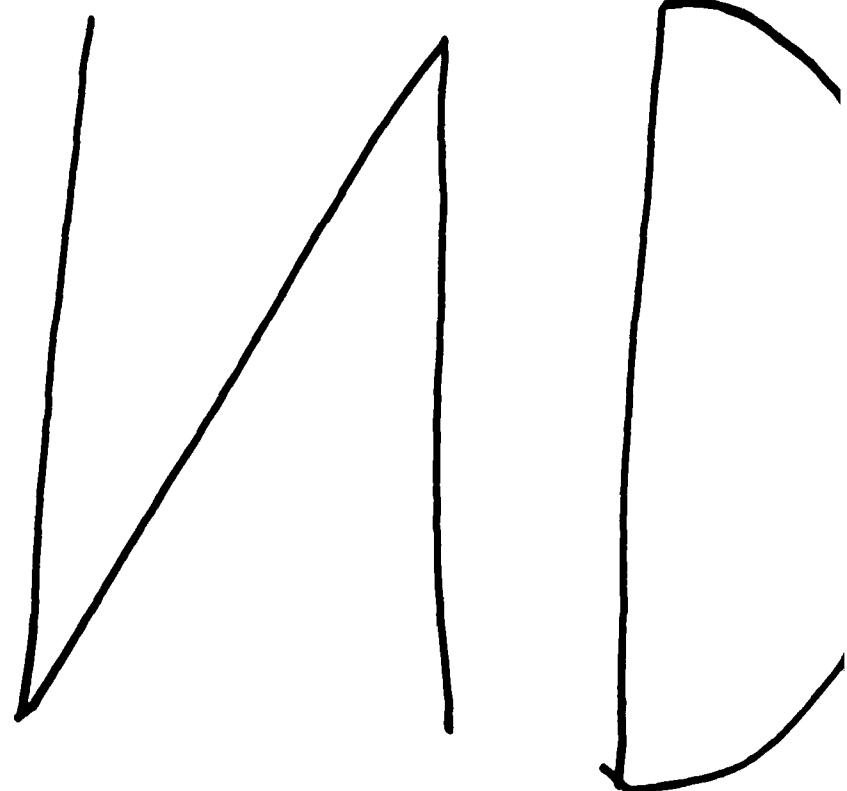
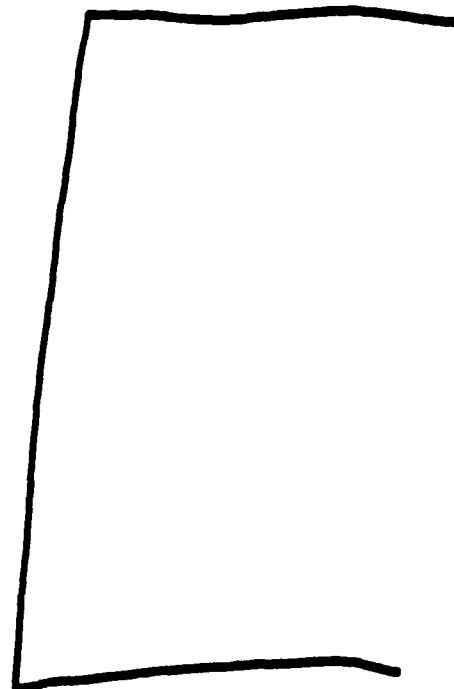
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